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<p>(54) Title: TOXIN ASSAY</p> <p style="text-align: center;">ASSAY FOR BOTULINUM TOXIN</p> <p>The diagram illustrates the assay process. A substrate peptide, represented by the sequence CCLNKWWTYKRLKAASFEFQSAGACLADARDDESL, is shown. It reacts with Botulinum Toxin (BoNT/B), depicted as a dimeric protein. The resulting complex then interacts with antibodies (labeled 'enz') to produce a 'Coloured Product'.</p> <p>(57) Abstract</p> <p>A toxin assay that uses a substrate for cleavage by the toxin and antibodies that do not recognise the substrate but recognise and bind to the product of cleavage of the substrate by the toxin. The substrate can be a nerve cell peptide when the assay is for botulinum toxin or tetanus toxin.</p>		

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TOXIN ASSAY

This invention relates to assays for toxins, in particular botulinum neurotoxins and tetanus toxins, that have peptidase activity. The invention also relates to antibodies useful in the assays and to solid-phase peptides useful in the assays.

The botulinum neurotoxins are a family of structurally similar, but antigenically different protein neurotoxins which act on the peripheral nervous system to block neuromuscular transmission. These neurotoxins are extremely potent, with a human lethal dose in the order of micrograms, and give rise to the rare but frequently fatal disease, botulism. Assays for the botulinum neurotoxins are currently used in both the food and pharmaceutical industry. The food industry employs assays for the botulinum neurotoxins to validate new food packaging methods and to ensure food safety. With the growing clinical use of the botulinum toxins, the pharmaceutical industry requires accurate assays for these toxins for both product formulation and quality control.

It is known to assay for botulinum toxin in foodstuffs using the mouse lethality test. This test has been the industry standard for many years, though over the past 10 years a number of immunoassay methods have been developed in an attempt to replace the mouse test in the majority of applications.

One such assay operates by addition of a test sample to a plate or column to which is attached an antibody that binds to toxin present in the sample. A further antibody is typically used to detect bound toxin. These enzyme-linked immunoassays (ELISA) have the advantages that they are specific to one botulinum toxin type and can be performed rapidly, in less than 2 hours. The ELISAs, however, suffer from several drawbacks:-

- (a) They do not measure the biological activity of the toxins,
- (b) They cannot distinguish between active and inactive toxin, and
- (c) Due to antigenic variations some toxins are not detected by these assays which therefore give rise to false negatives.

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The botulinum neurotoxins have recently been shown to possess highly specific zinc-endopeptidase activities within their light sub-units. Depending on the neurotoxin type these act to cleave small proteins within the nerve cell which are involved in neurotransmitter release. Botulinum types A and E toxins cleave protein SNAP-25. Botulinum types B, D, F and G and tetanus toxins cleave vesicle-associated membrane protein (VAMP-also called synaptobrevin). Botulinum type C toxin cleaves the protein syntaxin.

In the development of further toxin assays, various procedures have been devised for the evaluation of endopeptidase activities. Liquid chromatography procedures are known and are based on resolution of the peptide product and subsequent evaluation. These procedures are time-consuming, expensive and do not lend themselves readily to automation. It is also known to use spectrophotometric methods, requiring the development of suitable chromogenic peptide reagents. Such methods provide a continuous precise assay for endopeptidases. Spectrophotometric methods, however, require relatively pure preparations of enzyme and are not normally suitable for evaluation of endopeptidase activities in crude or particulate samples.

Despite these efforts, at present, the only convenient assay for the biological activity of the botulinum neurotoxins, and the only assay that is FDA approved, remains the mouse lethality test. This test suffers from a number of drawbacks:-

- (a) It is expensive and uses large numbers of laboratory animals,
- (b) It is non-specific unless performed in parallel with toxin neutralization tests using specific anti-sera, and
- (c) It is not very accurate unless large animal groups are used.

The present invention describes a novel assay system for toxins, using novel reagents. The assay aims to overcome or at least mitigate many of the drawbacks of present *in vitro* assays for these toxins.

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Accordingly, a first aspect of the invention provides a toxin assay comprising the steps:-

- (a) Combining a test compound with (i) a substrate that has a cleavage site for the toxin and (ii) antibodies that bind to the cleaved substrate but not to uncleaved substrate, and
- (b) Testing for the presence of antibodies bound to the cleaved substrate.

This has the advantage that the assay is capable of distinguishing between active and inactive toxin - as inactive toxin will have reduced or no activity. Also, antigenic variation between toxins of the same group will not significantly affect the working of the assay, because it is toxin activity (ability to cleave the substrate) that is being measured and not the precise antigenic make-up of the toxin.

In use of an embodiment of the invention, cleavage of the substrate by toxin present in the test compound generates a product that is recognised by and binds to the antibodies of the assay, which antibodies do not bind to the uncleaved substrate. It is preferred that the toxin cleaves the substrate at a single location so as to generate two products, one of which binds to the antibodies.

In another embodiment of the invention, the substrate is a peptide or protein that contains a cleavage site for the toxin and cleavage of the substrate generates new peptides having N- and C- terminal ends. The antibody binds to one of these newly formed peptides. A particular assay of the invention is for assay of a botulinum toxin or tetanus toxin and the assay determines whether amounts of one of these toxins are present in the test compound. In this particular assay, the substrate of the invention is a peptide or a protein that is cleaved by endopeptidase activity of the botulinum toxin or tetanus toxin. An advantage of the assay is that it distinguishes between active and inactive

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toxin, as the assay is a measurement of the peptidase activity present in the sample.

A further embodiment of the assay of the invention comprises:-

- (i) combining the test compound with a solid phase comprising a peptide selected from VAMP, SNAP-25, syntaxin and fragments thereof,
- (ii) washing the test compound from the solid phase,
- (iii) combining the solid phase with an antibody adapted for binding selectively with peptide cleaved by toxin, and
- (iv) detecting conjugates of the antibody with cleaved peptide.

Preferably, the antibody is adapted to bind selectively to a peptide selected from SEQ ID NO:s 1-7.

Another embodiment of the assay comprises:-

- (i) adding a test solution to an assay plate comprising immobilized peptide, the peptide being selected from VAMP, SNAP-25, syntaxin and fragments thereof,
- (ii) incubating the assay plate,
- (iii) washing the plate with a buffer,
- (iv) adding to the plate an antibody solution, said solution comprising an antibody adapted selectively to bind to a peptide selected from (1) a peptide the C-terminal end of which is selected from SEQ ID

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NO:s 1, 3 and 5; and (2) a peptide the N-terminal end of which is selected from SEQ ID NO:s 2, 4 and 6,

- (v) incubating the assay plate,
- (vi) washing the plate with a buffer, and
- (vii) measuring the presence of antibody on the assay plate.

In steps (ii) and (v) a suitable incubation period is from twenty minutes to 3 hours, at 35-39°C.

In use, the antibody can be linked to an enzyme and antibody on the plate measured by adding a substrate and observing conversion into a detectable, e.g. coloured, product.

It has already been noted that the assay of the invention detects active toxin in a compound under investigation. Some toxins, notably botulinum toxins, exist also in an inactive conformation, and as such would not be identified by the assay. A further embodiment of the assay includes pre-treatment of the test compound, to convert inactive toxin into active toxin. This is achieved for example by a protease, such as trypsin.

It is further preferred that the assay incorporates a substrate attached to a solid phase, for example covalently linked, such as via a terminal cysteine residue, to a solid phase of the assay.

The assay procedure of the invention thus typically represents a solid-phase microtitre based assay which can be applied to a number of specific toxins such as the botulinum neurotoxins and tetanus toxin. In contrast to spectrophotometric assays, the procedure of the invention can be easily automated and can be applied to very crude or particulate preparations of toxin

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endopeptidases. The procedure is also relatively inexpensive and easy to use.

For detection of antibodies bound to the cleaved substrate it is optional for the antibodies to be linked to a marker molecule or an enzyme. In an example, the marker is horse radish peroxidase which can be detected using conventional and well-known techniques.

In a particular embodiment of the invention, synthetic peptide substrates for the botulinum toxins (derived from sequences of intracellular proteins that are targets for the toxins) are prepared and used as the solid-phase endopeptidase substrate component in an assay system that follows the procedure:-

Step 1 - test solutions containing botulinum toxin are incubated with the solid - phase endopeptidase substrate. This results in the cleavage of the peptide at a specific point in the sequence which is dependant on the toxin type (this is illustrated in fig. 1).

Step 2 - incubation with anti-peptide antibody reagent. This antibody reagent is specific to one of the newly cleaved N- or C- terminal ends of the solid-phase peptide and does not recognise the intact peptide. The antibody reagent is covalently conjugated to an enzyme marker, such as peroxidase or alkaline phosphatase. It is a further option to have present two separate antibodies that bind, respectively, one to the N-terminal ends and the other to the C-terminal ends of the newly cleaved peptides.

Step 3 - incubation with substrate for the enzyme marker or further amplification by commercially available techniques.

It is seen that the assay of this latter embodiment has two main components.

The first component is an endopeptidase substrate component which contains or consists of a protein or peptide that is cleaved by the toxin endopeptidase to

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be assayed. This will typically be incorporated into the solid-phase component of the assay.

The endopeptidase substrate component may be, for example, the protein target of the endopeptidase (such as syntaxin; VAMP or SNAP-25) or a fragment of these proteins that is cleaved by the toxin endopeptidase. The endopeptidase substrate component may alternatively be a fusion protein comprising the above linked to another protein.

The solid-phase may be an ELISA plate, bead, dip-stick or any other matrix that can be used for immobilizing the endopeptidase substrate component. The solid-phase component may also be a nitrocellulose membrane or the equivalent as used in the Western blotting technique.

The second component is the antibody component which is an antibody raised against a short polypeptide (typically 6-8 amino acid residues in length) representing the sequence of either the newly generated N- or the newly generated C-terminal peptide obtained after cleavage of the endopeptidase substrate component by the toxin endopeptidase. The antibody has the properties that it does not recognise the endopeptidase substrate component but instead recognise only the cleaved peptide product.

The antibody may be linked to an enzyme, or a radioactive or fluorescent marker. Alternatively, the antibody may be the free protein and a second anti-antibody used in the assay procedure. The antibody may further be polyclonal or monoclonal in make up, an antibody fragment, or an antibody or antibody-fragment fused or linked to another protein.

In a second aspect of the invention there is provided an antibody for use in the assay of the first aspect characterised by the following properties:-

- (a) The antibody binds to a peptide that is the product of cleavage of a

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substrate by a toxin endopeptidase, and

- (b) The antibody does not bind to the uncleaved substrate.

In an embodiment of the second aspect, the antibody does not bind to the substrate, which is linked covalently or otherwise to a carrier molecule. It is preferred that the substrate is a peptide, more preferably a nerve cell peptide, that is cleavable by a toxin according to the first aspect of the invention. The peptide is preferably selected from syntaxin, VAMP and SNAP-25.

It is further preferred that the peptide substrate is cleaved by the toxin endopeptidase at a limited number of locations, and preferably at two, or more preferably one, location so as to generate a limited number of cleavage products, and that the antibody binds selectively to one of these products.

In preferred embodiments of the invention the antibody recognises one of the following peptide sequences:-

- | | | |
|----|---------------------|---------------|
| 1. | KAASSEF-n terminal | (SEQ ID NO:1) |
| 2. | LQAGASQ-c terminal | (SEQ ID NO:2) |
| 3. | RIDEANQ-c terminal | (SEQ ID NO:3) |
| 4. | GLMKTAR-n terminal | (SEQ ID NO:4) |
| 5. | QNRQIDR-c terminal | (SEQ ID NO:5) |
| 6. | SDAKEMI-n terminal. | (SEQ ID NO:6) |
| 7. | KAASTEf-n terminal. | (SEQ ID NO:7) |

In addition, the preferred antibodies do not recognise the one of the sequences 1-7 immediately above when that sequence is part of an intact, uncleaved peptide and that sequence does not contain the free N- or C-terminal ends shown immediately above.

An antibody according to the second aspect of the invention and for use in a toxin assay is prepared, for example, by the procedure:-

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- identifying a peptide that is cleaved by the toxin to generate a cleavage product,
- immunizing an animal, such as a rabbit, with the cleavage product,
- isolating antibodies to the cleavage product from the animal, and
- checking that the antibodies do not cross react with the peptide.

Another aspect of the invention provides a method of obtaining an antibody, the antibody being for use in a toxin assay, the method comprising identifying a macromolecule that is cleaved by a toxin into cleavage products, immunizing an animal against one of the cleavage products, isolating antibodies that bind to said one of the cleavage products and checking the antibody does not cross-react with the macromolecule. An embodiment of this method comprises:-

- (i) identifying a macromolecule that is cleaved by a toxin, thereby forming at least first and second cleavage products,
- (ii) immunizing an animal with a selected one of the cleavage products,
- (iii) isolating from the animal an antibody that binds to the selected cleavage product, and
- (iv) checking the antibody does not bind to the macromolecule.

The macromolecule is preferably a peptide or a protein. It is particularly preferred that the peptide or protein is cleaved into two fragments.

The method enables production of antibodies that possess a surprising and advantageous property, in particular for performing assays for toxin. The property of interest is the binding affinity for the shortened cleavage product, enabling binding to this product, uncontaminated by an affinity of any significance to the intact, uncleaved macromolecule.

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In another embodiment, the method comprises:-

- (i) identifying a peptide or protein that is cleaved by a toxin, thereby forming at least first and second cleavage products,
- (ii) immunizing an animal with an antigen selected from (1) a terminal fragment of the first cleavage product, (2) a synthetic fragment of the first cleavage product, (3) an analogue of (1) and (4) a carrier molecule linked to any of (1)-(3),
- (iii) isolating from the animal an antibody that binds to the first cleavage product.

Many carrier molecules are known in the art, and preferred carriers include keyhole limpet hemocyanin, bovine serum albumin and ovalbumin.

In a further aspect the invention provides a component of a toxin assay which is a peptide substrate attached to a solid phase of a toxin assay, the peptide substrate being a target for cleavage by toxin endopeptidase. Preferably, the peptide is selected from the intact peptide and fragments of:-

1. VAMP
2. SNAP-25
3. Syntaxin.

Particularly preferred target peptide sequences for use in the assay are selected from SEQ ID NOs. 8-11 illustrated below.

SEQ ID NO. 8 (VAMP isoform-1, aas 33-94):-

QQIQAQVDEVVDIMRVNVDKVLERDOKLSELDDRADALQAGASQFESSAA
KLKRKYWWKNLK

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SEQ ID NO. 9 (VAMP isoform-1 aas 60-94):-

LSELDDRADALQAGASQFESSAAKLKRKYWWKNLK

SEQ ID NO. 10 (SNAP-25 aas 137-206):-

VTNDARENEMDENLEQVSGIIGNLRHMAIDMGNEIDTQNRQIDRIMEKADSN
KTRIDEANQRATKMLGSG

SEQ ID NO. 11 (VAMP isoform-2, aas 60-94):-

LSELDDRADALQAGASQFETSAAKLKRKYWWKNLK

Further preferred peptides of the invention are peptides that include a sequence selected from SEQ ID NO:s 1-7.

The solid-phase peptides of the assay are stable and suitable for incorporation into test kits that provide for convenient and efficient toxin assays.

It would be appreciated by a skilled reader that the assay of the invention can also be performed with a solid phase that comprises antibody according to the second aspect of the invention. With this arrangement, the assay is performed by combining the toxin substrate with the test compound that possibly contains toxin. This mixture is then combined with the solid-phase antibody and thereafter the presence of cleaved peptide bound to the solid-phase antibody is detected. In an embodiment of the invention, the cleaved peptide bound to antibody is detected using radiolabelled peptide or, alternatively, a further peptide specific antibody. Accordingly, a fourth aspect of the invention provides a solid-phase antibody for use in a toxin assay wherein the antibody has the binding properties according to the second aspect of the invention.

The assay procedures of the invention thus encompass various features:-

- (i) The production of an antibody to a polypeptide sequence (e.g. XXXXN) which is recognised only when amino acid N is free and

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not when the same sequence makes up a longer peptide (e.g. XXXXNXXXX). It is surprising that suitable antibodies can be raised that show non-reactivity to the longer peptide.

- (ii) The use of the properties of such an antibody in a rapid, solid-phase assay for toxin endopeptidase. In the example of the botulinum or tetanus neurotoxin assay, the procedure exploits the zinc-endopeptidase activities of these toxins.
- (iii) An assay that uses toxin target molecules, such as nerve cell peptides, as solid-phase assay components.
- (iv) An assay that uses the antibodies described in (i) as solid-phase assay components.

A feature of the invention is that antibodies used to detect cleaved peptide do not bind to uncleaved peptide; for, clearly, such binding would generate false positives and compromise assay accuracy. A convenient means of determining that antibodies do not cross-react with intact peptide is to screen antibodies using two plates. The first contains intact peptide attached to a solid phase. The second contains cleaved peptide attached to a solid phase, a free end of the cleaved peptide being, for example, one of SEQ ID NO:s 1-7 or another sequence recognised by assay antibodies.

Antibodies under screening are added to both plates, allowed to incubate for a period and the plates are then washed and subsequently tested for the presence of bound antibody. Suitable antibodies, i.e. non cross-reacting ones, generate a large signal on the second plate, indicating the presence of bound antibody and a weak signal, preferably virtually no signal, on the first plate.

If a number of potential assay antibodies are tested then it is straightforward to compare the ratio of signal (second plate result) to noise (first plate result) so that antibodies are selected with a high selectivity for cleaved peptide over intact. In an embodiment of the invention, this ratio is at least 10:1, preferably at least 20:1 and more preferably at least 50:1. A particularly preferred ratio

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would be 100:1 or higher, reflecting an affinity for cleaved peptide at least 2 orders of magnitude greater than affinity for intact peptide.

The invention is of advantage in that it provides an assay that measures biological activity of the toxins, e.g. botulinum neurotoxin, tetanus toxin, and with a sensitivity comparable to that of a conventional ELISA system. The inventors have observed that the assay is capable of detecting less than 1ng of toxin per ml in the case of botulinum A and B toxins. For many workers, accurate quantification of toxin would be highly desirable for use in risk assessment. Also, false negatives are unlikely as the assay is a direct measurement of toxin endopeptidase activity. The observations by the inventors are that assay noise levels in serum and other samples are significantly lower than those obtainable in conventional immunoassay systems. The inventors have to date had no false negatives or false positives with the assay for botulinum B toxin and the noise levels were lower in toxin samples containing serum than the noise levels that can be obtained with a conventional ELISA. Further, the peptide solid-phase assay component is stable with a very long shelf life.

The assay and assay components of the invention described are particularly sensitive for toxin and have been found by the inventors to have comparable and improved sensitivity to ELISAs for toxin. A further advantage of the invention is that it has potential to be more sensitive, as further specific antibodies are identified for use in the assay. Contrast this situation with that for ELISAs, which rely on antibody affinity for toxin. As this affinity is already optimal due to extensive work in isolating high affinity antibodies, few increases in ELISA sensitivity are expected.

There now follows a number of examples of the invention illustrated by drawings in which:-

Fig. 1 shows a schematic representation of a botulinum toxin

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- assay;
- Figs. 2-4 show results achieved using the botulinum toxin assay of Example 1;
- Figs. 5-6 show results in which the botulinum toxin assay of Example 1 is further improved and made more sensitive by signal amplification and toxin activation procedures described in Example 2; and
- Fig. 7 shows results achieved using the botulinum toxin assay of Example 4.

Example 1. An Assay for Botulinum Type B Neurotoxin (BoNT/B)

For the botulinum type B assay a peptide representing the sequence of VAMP isoform-1, residues 60-94 (SEQ ID NO:9, plus an C-terminal cysteine residue) was used:

LSELDDRADALQAGASQFESSAAKLKRKYWWKNLKC

Microtitre assay plates were prepared as follows:- The above peptide was diluted to a final concentration of $10 \mu\text{gml}^{-1}$ in 0.05 M sodium phosphate buffer, pH 6.5 containing 1mM EDTA and added (100 μl /well) to a Sulphydryl Binding Plate (Costar). After incubation for 1 hour at room temperature the peptide solution was removed and the plates washed 3 times with phosphate buffered saline (PBS), pH 7.4 (no detergent is used at this stage in the washing). Any remaining binding sites on the plates were then blocked by the addition (100 μl /well) of a PBS buffer containing 0.1% Tween 20 and 5% Foetal bovine serum. The plates were then incubated for one hour at 37°C with continuous shaking after which the blocking solution was removed.

Assay of *Clostridium botulinum* type B neurotoxin:- Toxin solutions were diluted in an assay buffer (e.g. 0.05M Hepes buffer, pH 7.4 containing 10 μM ZnCl_2 and 1% foetal bovine serum) containing 10 mM dithiothreitol (or an equivalent reagent to

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provide reducing conditions for the endopeptidase activity of the toxins) and added (100 μ l/well) to peptide coated microtitre plates. The plate is then incubated for one hour at 37°C with continuous shaking. After this period in which BoNT/B cleaves the peptide as depicted in Fig 1, the plates were washed 3 times with PBS containing 0.1% Tween 20.

Antibody specific to the newly cleaved peptide is then added. In this example of the assay, the antibody is specific to the following cleaved sequence of VAMP (SEQ ID NO:1 plus C-terminal cysteine):-

CKAASSEF-NH₂

The antibody (diluted in PBS buffer containing 0.1% Tween 20 and 5% foetal bovine serum) is added (100 μ l/well) and incubated for one hour at 37°C with continuous shaking. The plates were then washed 3 times with PBS containing 0.1% Tween 20.

If the antibody is conjugated to peroxidase, appropriate peroxidase substrates are added at this stage to develop the colour and provide the results of the assay.

An alternative is that the antibody is in its free form, in which case the addition of a commercially available second antibody is required. If, for example, the anti-peptide antibody is raised in guinea-pigs, then this second antibody is an anti-guinea-pig peroxidase conjugate. The second antibody conjugate (diluted in PBS buffer containing 0.1% Tween 20 and 5% foetal bovine serum) is added (100 μ l/well) and incubated for one hour at 37°C with continuous shaking. After washing off the second antibody (x3 with PBS containing 0.1% Tween 20), appropriate peroxidase substrates may be added at this stage to develop the colour and provide the results of the assay.

Figure 2 illustrates the results of a typical assay for BoNT/B using the two antibody detection system described above. The data illustrate the use of two different

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VAMP peptides as the solid-phase peptide in the assay. The sequences of these peptides near the cleavage site for BoNT/B represent the two isoforms of human VAMP.

VAMP-1 (60-94) LSELDDRADALQAGASQFESSAAKLKRKYWWKNLKC
(SEQ ID NO:9, plus C-terminal cysteine)

VAMP-2 (60-94) LSELDDRADALQAGASQFETSAAKLKRKYWWKNLKC
(SEQ ID NO:11, plus C-terminal cysteine)

The data show that, at an arbitrary cut-off point of 0.3 absorbance units above background, the sensitivity for the detection of BoNT/B in these assay was approximately 1ng/ml using the VAMP-1 peptides as the solid-phase and approximately 5ng/ml using the VAMP-2 peptides as the solid-phase.

Figure 3 illustrates the specificity of the assay system for BoNT/B. Zero absorbance was recorded for (1) BoNT/F, (2) Tetanus toxin and (3) BoNT/B plus EDTA. These data show that an assay designed to detect BoNT/B did not give false-positive results in the presence of the closely related botulinum type F and tetanus toxins. The data also show that no signal was obtained in assays performed with BoNT/B in the presence of EDTA (which is a metal ion chelating agent). The latter result illustrates that the assay is dependent on the zinc-metalloprotease activity of BoNT/B.

Figure 4 illustrates the assay kinetics of solid-phase peptide cleavage by BoNT/B using both the single antibody (i.e. anti-peptide antibody directly conjugated to peroxidase) and dual antibody (i.e. in which the free anti-peptide antibody is used in conjunction with a anti-species peroxidase conjugate) detection systems. The concentration of BoNT/B was fixed at 100ng/ml and the assay signal measured at various times. The data show that the dual antibody assay system provides the more rapid and sensitive means with which to measure solid-phase peptide cleavage by BoNT/B.

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Example 2. Amplification and Enhancement of Assays for the Botulinum Neurotoxins.

There are a number of ways in which the assays for the botulinum toxins such as that described in Example 1 may be further enhanced to provide greater sensitivity:-

(a) Further amplification of the assay signal using commercially available amplification systems such as the ELASTTM (Dupont) system.

(b) Limited trypsin treatment of the neurotoxin prior to assay. All of the botulinum neurotoxins are produced, in the bacterium, as single polypeptide chains which are then subsequently activated by specific bacterial proteases to give the active, di-chain form of the toxin. In some instances, notably in the case of botulinum neurotoxin types B and E, the toxins are not fully activated and can contain up to 100% inactive neurotoxin. These inactive, single chain, forms of the toxin express the endopeptidase activity of the toxin either weakly or not at all. Limited trypsin treatment of the botulinum neurotoxins converts the single chain toxin form to the di-chain, active toxin form. Thus, in botulinum neurotoxin types which contain a portion of single chain toxin, trypsin treatment results in both an increase in the endopeptidase activity and specific toxicity of the neurotoxin.

Figure 5 illustrates the increase in sensitivity of an assay for BoNT/B when an amplification system such as the ELAST system is applied to a typical assay as described in Example 1. The data show that, at an arbitrary cut-off point of 0.3 absorbance units above background, the sensitivity for the detection of BoNT/B in the non-amplified assay was approximately 1ng/ml while that in the amplified assay system was almost 10-fold higher giving a sensitivity close to 0.1ng/ml.

The results show that the assay of the invention can be readily used in conjunction with commercially available amplification systems to provide improved sensitivity.

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Figure 6 illustrates the effect of limited trypsin treatment of BoNT/B on the sensitivity of the neurotoxin assay. BoNT/B samples (1mg/ml) were treated with trypsin (final concentration of 2.5µg/ml) for 30min at 37°C and the reaction stopped using a 5-10 molar excess of trypsin inhibitor. Figure 6 shows that, for both the amplified and non-amplified forms of the assay (see Fig.), the effect of the trypsin treatment is to increase the sensitivity of the assay system for BoNT/B.

An alternative strategy for the assay for BoNT/B is performed using a different combination of solid-phase peptide substrate and specific antibody to cleaved sequences. In this aspect of the assay the solid-phase VAMP peptide is attached by an N-terminal cysteine residue (SEQ ID NO:9, plus N-terminal cysteine):-

CLSELDDRADALQAGASQFESSAAKLKRKYWWKNLK

The specific antibody to the cleaved sequence in this embodiment of the invention is raised against the following cleaved sequence of VAMP:-

LQAGASQ-COOH
(SEQ ID NO:2)

Example 3. An Assay for Tetanus Toxin

Tetanus toxin cleaves the protein VAMP at an identical site to that of BoNT/B. The specificities of the endopeptidase activities of the two toxins however differ in the minimum peptide substrate size required for cleavage. While BoNT/B requires peptide substrates of 30-35 residues in length for optimal cleavage, the requirement for tetanus toxin is peptide substrates of >50 residues in length.

Tetanus toxin cleavage assays are therefore carried out in an identical manner to those for BoNT/B with the exception that the solid-phase peptide is a peptide of human VAMP-1 isoform-1, residues 33-94 (SEQ ID NO:8). Example of the invention described for BoNT/B are equally applicable to tetanus toxin.

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Example 4. An Assay For Botulinum Type A Toxin (BoNT/A)

For the botulinum type A neurotoxin assay, microtitre plates are coated (10µg/ml) with the following peptide representing a sequence taken from protein SNAP-25 (aas 137-206).

VTNDARENEMDENLEQVSGIIGNLRHMALDMGNEIDTQNRQIDRIMEKADSN
KTRIDEANQRATKMLGSG (SEQ ID NO:10)

Toxin test solutions buffered with 0.05M HEPES pH 7.2 containing 10µM ZnCl₂ and 10mM 2-mercaptoethanol are then added and the plate incubated at 37°C for 1h.

After washing the plates are then incubated with anti-peptide antibody conjugated to horse radish peroxidase. The antibody is specific to either of the following cleaved sequences of SNAP-25:-

RIDEANQ-COOH (SEQ ID NO:3)

GLMK TAR-NH₂ (SEQ ID NO:4)

After incubation the excess peptide is removed by washing and then the peroxidase substrates added to develop the colour.

Figure 7 shows the results of a typical assay for BoNT/A. In this assays system the solid-phase peptide was SNAP-25 (137-206) as depicted above, used in conjunction with antisera specific to the cleaved sequence of SNAP-25:-

RIDEANQ-COOH (SEQ ID NO:3)

Assay protocol was as described for BoNT/B in the Example 1. The assay detected BoNT/A at a concentration of approximately 1ng/ml (using an arbitrary absorbance

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cut-off point of 0.3 units above background).

The amplification systems described in Example 1 may also be applied to assays for BoNT/A.

Example 5. An Assay Of Botulinum Type E Toxin

For the botulinum type E neurotoxin assay, microtitre plates are coated (10 μ g/ml) with the following peptide representing a sequence taken from protein SNAP-25 (aas 137-206).

VTNDARENEMDENLEQVSGIIGNLRHMAIDMGNEIDTQNRQIDRIMEKADSM
KTRIDEANQRATKMLGSG (SEQ ID NO:10)

Toxin test solutions buffered with 0.05M HEPES pH 7.2 containing 10 μ M ZnCl₂ and 10mM 2-mercaptoethanol are then added and the plate incubated at 37°C for 1h.

After washing the plates are then incubated with anti-peptide antibody conjugated to horse radish peroxidase. The antibody is specific to either of the following cleaved sequences of SNAP-25:-

QNRQIDR-COOH (SEQ ID NO:5)

SDAKEMI-NH₂ (SEQ ID NO:6)

After incubation the excess peptide is removed by washing and then the peroxidase substrates added to develop the colour.

Example 6. Preparation And Use Of Microtitre Plates And Other Assay Solid Phases

Microtitre plate or dip-sticks are prepared by a number of different methods:-

- 21 -

(a) Microtitre plates are coated (100 μ l/well) with the "endopeptidase peptide substrate" at a concentration of 10 or 20 μ g/ml in phosphate buffered saline pH 7.4 (or water) and incubated overnight at 4°C.

(b) The "endopeptidase peptide substrate" is produced with a cysteine residue at one end. The end of the peptide to which the cysteine residue is added is determined by the specificity of the antibody to be used in the assay. The peptide cleavage product (formed during the course of the assay) which is recognised by the specific antibody must also contain the cysteine residue at its distal end. The "endopeptidase peptide substrate" is diluted to a final concentration of 10 μ gml⁻¹ in 0.05 M sodium phosphate buffer, pH 6.5 containing 1mM EDTA and added (100 μ l/well) to a Sulphydryl Binding Plate (Costar). After incubation for 1 hour at room temperature the peptide solution is removed and the plates washed 3 times with phosphate buffered saline, pH 7.4 (no detergent is used at this stage in the washing).

(c) A cysteine containing derivative of the "endopeptidase peptide substrate" (as above) is linked to a carrier protein (e.g. maleimide-activated BSA) which is commercially available (Pierce Warriner, U.K.) which is then bound to microtitre plates as described in (a) above.

(d) The "endopeptidase peptide substrate" is bound onto another solid-phase such as nitrocellulose membranes and procedures used similar to those defined in standard Western blotting techniques. The cleavage assay can be performed in several ways:-

- (i) The peptide substrate is cleaved on the nitrocellulose membrane.
- (ii) The peptide is cleaved by botulinum toxin in solution and then the products bound to the nitrocellulose membrane.
- (iii) The botulinum/tetanus toxin target proteins may be cleaved intracellularly (or *in vivo*) by the toxins, the cell extracts separated by SDS gel electrophoresis and then the cleaved peptide products transferred to nitrocellulose by Western blotting.

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The plates are then washed with phosphate buffered saline (PBS) containing 0.1% Tween 20 and then blocked (to prevent further binding of proteins to the polystyrene) with a solution of 5% foetal calf serum in PBS (or 1% BSA in PBS or other blocking cocktails such as powdered milk in PBS).

Plates in this condition are suitable to be stored frozen for many months (or even years).

To perform the assay, test solutions of toxin are buffered with 0.25M Hepes pH 7.4 buffer containing $10\mu\text{M}$ ZnCl_2 and 20mM 2-dithiothreitol (toxin solution: buffer ratio of 4:1) and then added to microtitre plates and incubated for 1hr at 37°C . The toxin test solution is then washed with PBS/tween and then an antibody-peroxidase conjugate added. (The antibody is specific to one exposed end of the cleavage product and is added at a pre-determined dilution depending on the batch). After incubation for 1hr at 37°C the antibody-peroxidase conjugate is washed off the plate with PBS/tween and then peroxidase substrates added to develop the colour.

Example 7. Method Of Specific Antibody Production

The peptides to which antibodies are to be raised are synthesised with a cysteine residue at the opposite end of interest. In Example 1, the two peptides synthesised are as follows:

CKAASSEF-NH₂ (SEQ ID NO:1, plus cysteine)

CLQAGASQ-COOH (SEQ ID NO:2, plus cysteine)

The peptide is then linked to a carrier protein (e.g. Keyhole Limpet Hemocyanin or Bovine Serum Albumin) via the cysteine residue. This is achieved by mixing the peptide with the maleimide-activated carrier protein (commercially available from Pierce Warriner, U.K.) as detailed by the suppliers.

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The conjugated peptide is then injected in the presence of an adjuvant (e.g. Freund's adjuvant) into animals (e.g. guinea-pigs or rabbits). One immunisation schedule is to give animals 50 μ g of the peptide-protein at times 0, 4 weeks and 8 weeks; animals are then bled after 10 weeks.

Antibody in serum is purified by any one of the published methods for purifying IgG from sera. Anti-peptide specific antibody is optionally further purified by affinity chromatography using the immunising peptide immobilised onto chromatography medium (kit and method commercially available from Pierce Warriner, U.K.).

Antibodies with the desired binding characteristics are assessed as describe in Example 6. The assay should not give a colour in the absence of toxin and should give a strong colour in the presence of 1 μ g/ml of the desired toxin.

Example 8. Quantification Of The Assay

The assay is made quantitative by incorporating a toxin standard. The colour produced by various concentrations of the toxin is measured by a microtitre plate reader and a standard curve produced from which the toxin concentration in unknown samples may be assessed.

* * * * *

Thus the invention provides a novel and efficient toxin assay and provides novel reagents for use in the assay. The assay is inexpensive compared to existing assays, for example those using a mouse lethality test, and is suitable for industrial application. Further advantages of the assay and its reagents have been described above.

- 24 -

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:

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(F) POSTAL CODE (ZIP): SP1 3QS

(ii) TITLE OF INVENTION: TOXIN ASSAY

- 25 -

(iii) NUMBER OF SEQUENCES: 11

(iv) COMPUTER READABLE FORM:

- (A) MEDIUM TYPE: Floppy disk
- (B) COMPUTER: IBM PC compatible
- (C) OPERATING SYSTEM: PC-DOS/MS-DOS
- (D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

(vi) PRIOR APPLICATION DATA:

- (A) APPLICATION NUMBER: GB 9411138.2
- (B) FILING DATE: 30-JUN-1994

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 7 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

Phe Glu Ser Ser Ala Ala Lys
1 5

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 7 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

Leu Gln Ala Gly Ala Ser Gln
1 5

- 26 -

(2) INFORMATION FOR SEQ ID NO: 3:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 7 amino acids
 - (B) TYPE: amino acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

Arg Ile Asp Glu Ala Asn Gln
1 5

(2) INFORMATION FOR SEQ ID NO: 4:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 7 amino acids
 - (B) TYPE: amino acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

Arg Ala Thr Lys Met Leu Gly
1 5

(2) INFORMATION FOR SEQ ID NO: 5:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 7 amino acids
 - (B) TYPE: amino acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

Gln Asn Arg Gln Ile Asp Arg
1 5

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 7 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

Ile Met Glu Lys Ala Asp Ser
1 5

(2) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 7 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

Phe Glu Thr Ser Ala Ala Lys
1 5

(2) INFORMATION FOR SEQ ID NO: 8:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 62 amino acids

- 28 -

- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

Gln Gln Thr Gln Ala Gln Val Asp Glu Val Val Asp Ile Met Arg Val

1 5 10 15

Asn Val Asp Lys Val Leu Glu Arg Asp Gln Lys Leu Ser Glu Leu Asp

20 25 30

Asp Arg Ala Asp Ala Leu Gln Ala Gly Ala Ser Gln Phe Glu Ser Ser

35 40 45

Ala Ala Lys Leu Lys Arg Lys Tyr Trp Trp Lys Asn Leu Lys

50 55 60

(2) INFORMATION FOR SEQ ID NO: 9:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 35 amino acids
 - (B) TYPE: amino acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

Leu Ser Glu Leu Asp Asp Arg Ala Asp Ala Leu Gln Ala Gly Ala Ser

1 5 10 15

- 29 -

Gln Phe Glu Ser Ser Ala Ala Lys Leu Lys Arg Lys Tyr Trp Trp Lys

20

25

30

Asn Leu Lys

35

(2) INFORMATION FOR SEQ ID NO: 10:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 70 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

Val Thr Asn Asp Ala Arg Glu Asn Glu Met Asp Glu Asn Leu Glu Gln

1

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10

15

Val Ser Gly Ile Ile Gly Asn Leu Arg His Met Ala Leu Asp Met Gly

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25

30

Asn Glu Ile Asp Thr Gln Asn Arg Gln Ile Asp Arg Ile Met Glu Lys

35

40

45

Ala Asp Ser Asn Lys Thr Arg Ile Asp Glu Ala Asn Gln Arg Ala Thr

50

55

60

- 30 -

Lys Met Leu Gly Ser Gly

65

70

(2) INFORMATION FOR SEQ ID NO: 11:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 35 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

Leu Ser Glu Leu Asp Asp Arg Ala Asp Ala Leu Gln Ala Gly Ala Ser

1

5

10

15

Gln Phe Glu Thr Ser Ala Ala Lys Leu Lys Arg Lys Tyr Trp Trp Lys

20

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30

Asn Leu Lys

35

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CLAIMS

1. An assay for a toxin comprising the steps:-
 - (a) combining a test compound with a substrate that has a cleavage site for the toxin and with antibody that binds to the cleaved substrate but not to uncleaved substrate, and
 - (b) testing for the presence of antibody bound to the cleaved substrate.
 2. An assay according to Claim 1 in which the toxin has endopeptidase activity.
 3. An assay according to Claim 1 or Claim 2 in which the toxin is a botulinum toxin or a tetanus toxin.
 4. An assay according to any of Claims 1-3 in which the substrate is a peptide or a protein which is cleaved by the toxin to generate new peptides having N- and C- terminal ends.
 5. An assay according to Claim 4 in which the peptide substrate is attached to a solid phase component of the assay.
 6. An assay according to any previous claim utilizing assay components (a) and (b):
 - (a) a peptide linked to a solid-phase, the peptide being cleavable by the toxin to generate a cleavage product,
 - (b) an antibody that binds to the cleavage product but not to the peptide,
- and wherein the assay comprises the steps of:
- (i) combining a test compound that may contain or consist of the toxin with the solid-phase peptide to form an assay mixture,

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- (ii) subsequently or simultaneously combining the assay mixture with the antibody, and
 - (iii) subsequently or simultaneously determining whether there has been formed any conjugate between the antibody and the cleavage product.
7. An assay according to Claim 6 for botulinum toxin or tetanus toxin in which step (i) is carried out in the presence of a zinc compound.
8. An assay according to Claim 7 in which the peptide is selected from intact peptides or fragments thereof selected from VAMP, SNAP-25 and syntaxin.
9. An assay according to Claim 8 comprising:-
- (i) combining the test compound with a solid phase comprising a peptide selected from VAMP, SNAP-25, syntaxin and fragments thereof,
 - (ii) washing the test compound from the solid phase,
 - (iii) combining the solid phase with an antibody adapted for binding selectively with peptide cleaved by toxin,
 - (iv) detecting a conjugate of the antibody with cleaved peptide.
10. An assay according to Claim 9 wherein the antibody is adapted to bind selectively to a peptide selected from SEQ ID NO:s 1-7.
11. An assay according to Claim 8 comprising:-
- (i) adding a test solution to an assay plate comprising immobilized peptide, the peptide being selected from VAMP, SNAP-25, syntaxin

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and fragments thereof,

- (ii) incubating the assay plate,
- (iii) washing the plate with a buffer,
- (iv) adding to the plate an antibody solution, said solution comprising an antibody adapted selectively to bind to a peptide selected from (1) a peptide the C-terminal end of which is selected from SEQ ID NO:s 1, 3 and 5, and (2) a peptide the N-terminal end of which is selected from SEQ ID NO:s 2, 4 and 6,
- (v) incubating the assay plate,
- (vi) washing the plate with a buffer,
- (vii) measuring the presence of antibody on the assay plate.

12. An assay according to Claim 11 wherein the antibody is linked to an enzyme and the presence of antibody on the plate is measured by adding an enzyme substrate and measuring the conversion of the substrate into detectable product.

13. An assay according to Claim 12 wherein the detectable product is coloured and is measured by absorbance at a selected wavelength.

14. An assay according to any previous claim comprising converting inactive toxin present in the test compound to active toxin.

15. An assay according to Claim 14 comprising adding a protease to the test compound.

16. An assay according to any of Claims 1-13 comprising detecting antibody-

- 34 -

peptide conjugate using a further antibody specific to the first antibody and linked to an enzyme.

17. A method of obtaining an antibody, the antibody being for use in a toxin assay, the method comprising identifying a macromolecule that is cleaved by a toxin into cleavage products, immunizing an animal against one of the cleavage products, isolating antibodies that bind to said one of the cleavage products and recovering antibody that does not cross-react with the macromolecule.

18. A method of obtaining an antibody according to Claim 17, the antibody being for use in a toxin assay, the method comprising:-

- (i) identifying a macromolecule that is cleaved by a toxin, thereby forming at least first and second cleavage products,
- (ii) immunizing an animal with a selected one of the cleavage products,
- (iii) isolating from the animal an antibody that binds to the selected cleavage product, and
- (iv) checking the antibody does not bind to the macromolecule.

19. A method according to Claim 18 comprising identifying a peptide or protein that is cleaved by a toxin.

20. A method according to Claim 19 comprising identifying a peptide or protein that is cleaved by a toxin at a single location, generating only two fragments.

21. A method of obtaining an antibody according to Claim 17, the antibody being for use in a toxin assay, the method comprising:-

- (i) identifying a peptide or protein that is cleaved by a toxin, thereby

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forming at least first and second cleavage products,

(ii) immunizing an animal with an antigen selected from (1) a terminal fragment of the first cleavage product, (2) a synthetic fragment of the first cleavage product, (3) an analogue of (1) and (4) a carrier molecule linked to any of (1)-(3),

(iii) isolating from the animal an antibody that binds to the first cleavage product.

22. A method according to Claim 21 comprising immunizing an animal with an antigen comprising a terminal fragment of the first cleavage product linked to a carrier protein selected from Keyhole Limpet Hemocyanin, Bovine Serum Albumin and Ovalbumin.

23. An antibody for use in the assay according to any of Claims 1-16 characterised in that it binds to a peptide that is the product of cleavage of a substrate by a toxin endopeptidase, and it does not bind to the uncleaved substrate.

24. An antibody according to Claim 23 that does not bind to a peptide selected from VAMP, SNAP-25 and syntaxin but does bind to a product of cleavage by the toxin of one of these peptides.

25. An antibody according to Claim 24 adapted to bind to a peptide selected from SEQ ID NO:s 1-7.

26. An antibody according to Claim 25 adapted to bind to an antigen of formula:-
P-Q-X

wherein X is a peptide selected from SEQ ID NO:s 1-7 and is covalently linked at one end to P-Q, and wherein P-Q is a carrier in which P is a carrier protein and Q is an amino acid or an amino acid sequence adapted to attach peptide X to carrier

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P.

27. A component for a toxin assay comprising a peptide substrate of the toxin linked to a solid-phase.

28. An assay component according to Claim 27 comprising a peptide selected from intact peptide and fragments of VAMP, SNAP-25 and syntaxin immobilized on a solid phase.

29. An assay component according to Claim 28 wherein the solid phase is selected from an assay plate, an assay well, a nitrocellulose membrane, a bead, a dipstick and a component of an elution column.

30. A toxin assay kit comprising (1) an assay component according to any of Claims 27-29, (2) (a) an antibody according to any of Claims 23-26 linked to or including an enzyme or (b) a first antibody according to any of claims 23-26 and a second antibody adapted to bind to the first antibody and linked to or including an enzyme, and (3) a substrate for the enzyme.

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ASSAY FOR BOTULINUM TOXIN

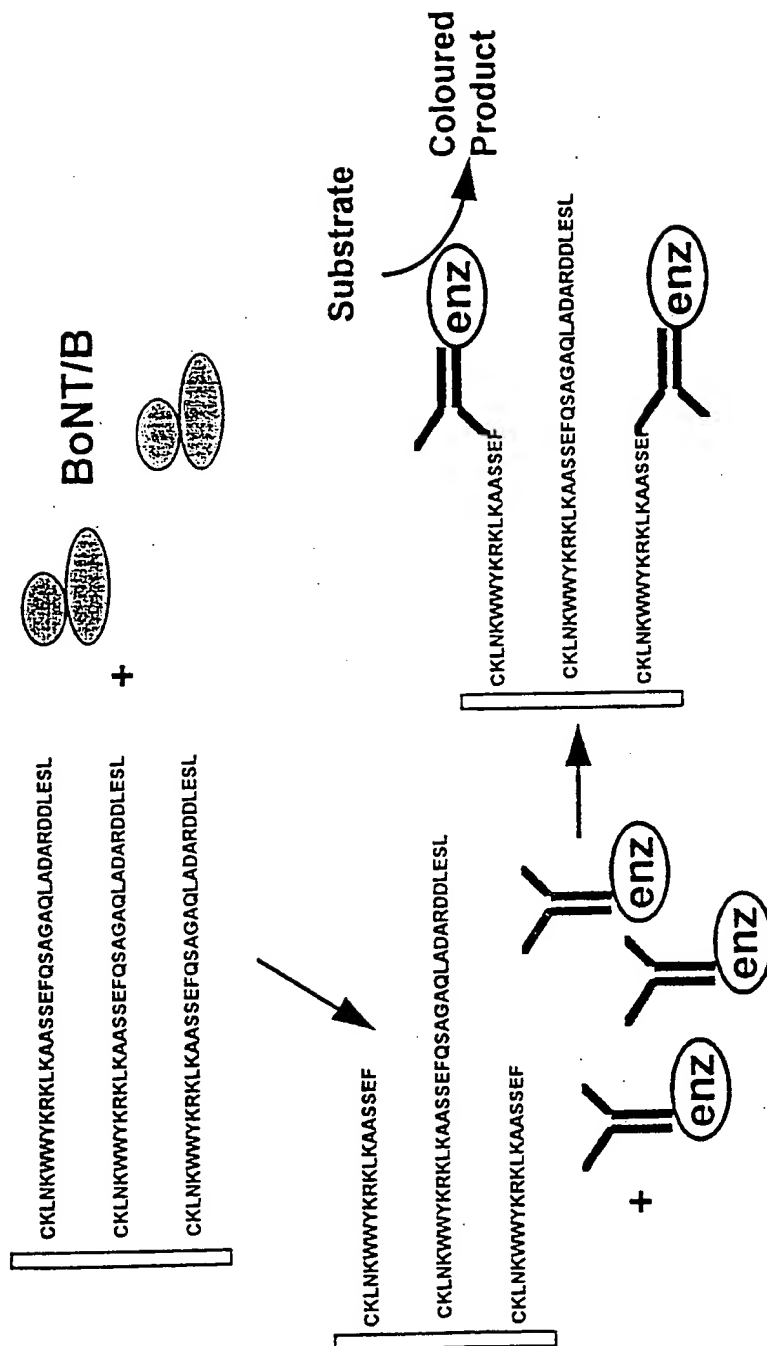


FIG. 1

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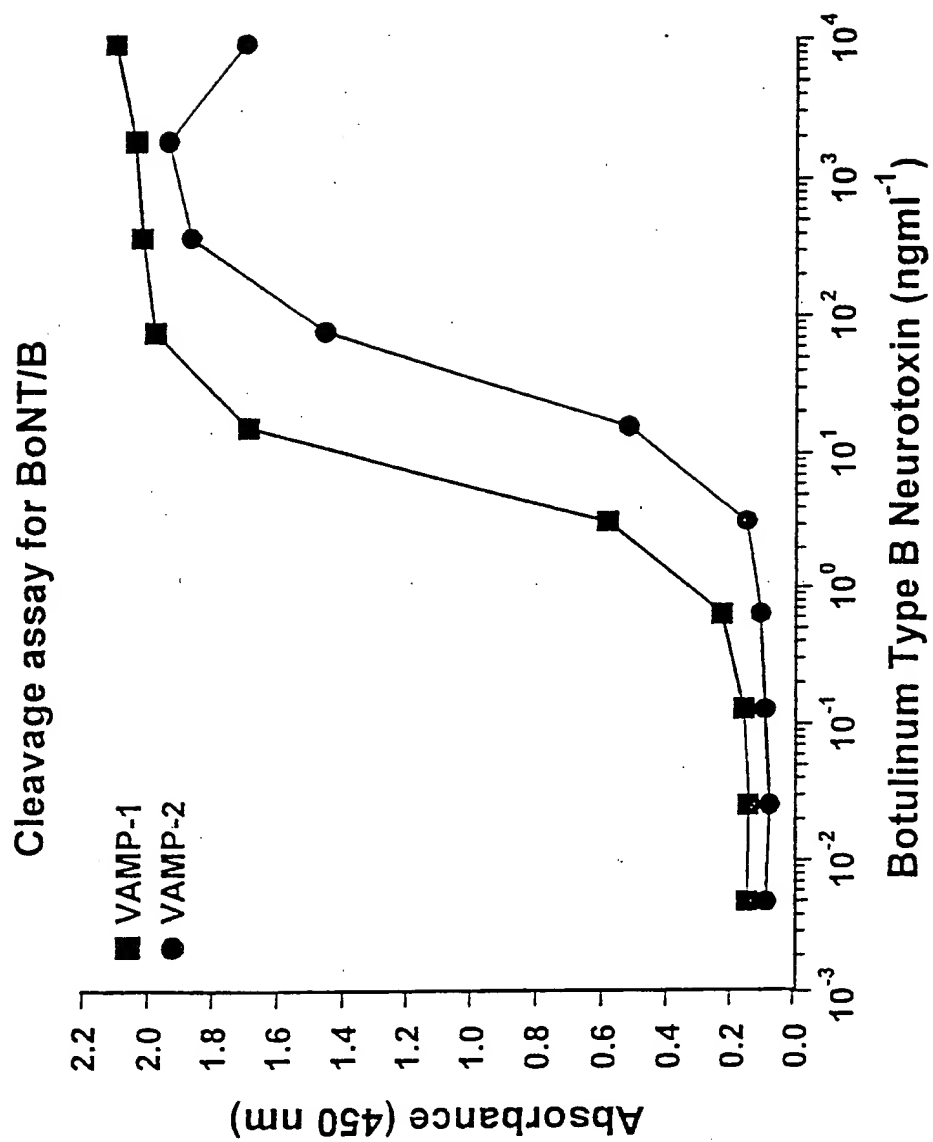


FIG.2

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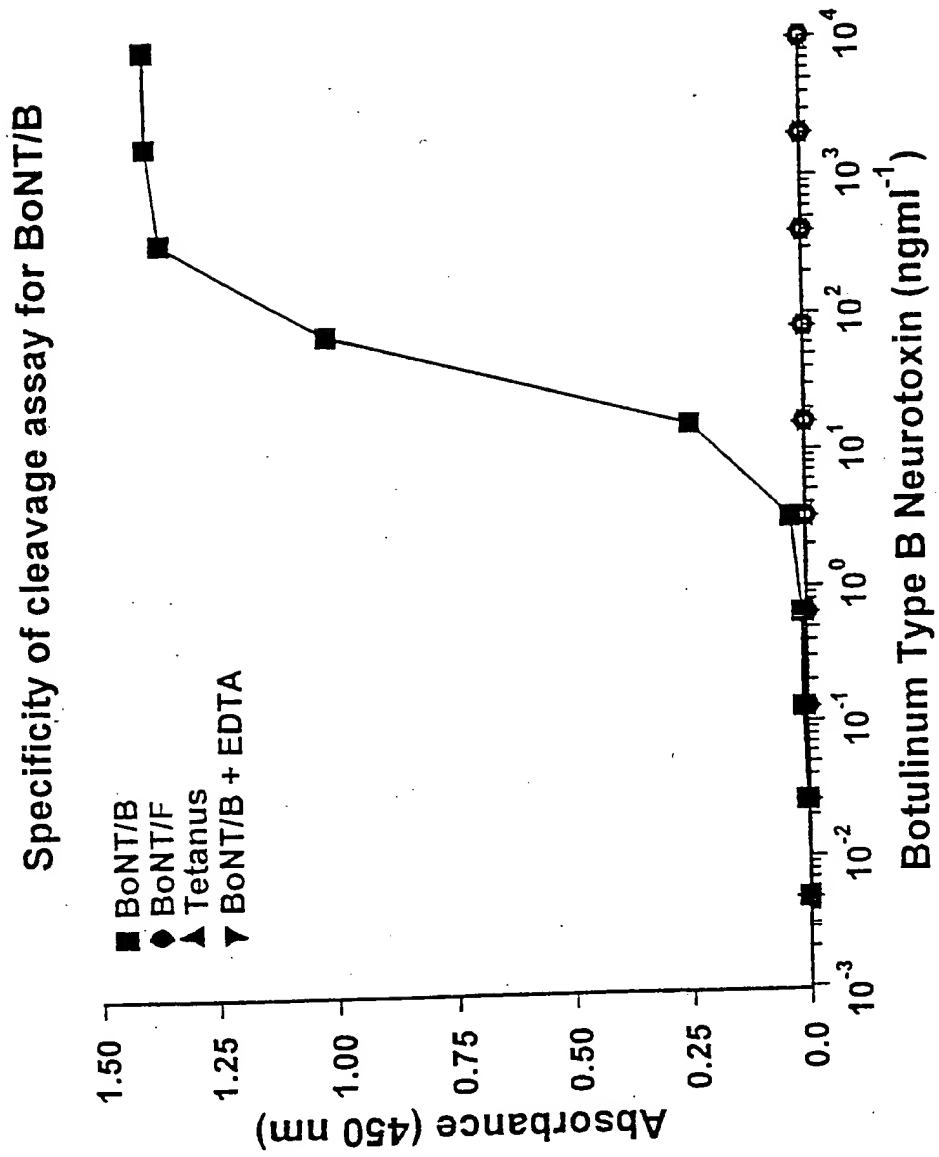


FIG. 3

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Kinetics of the cleavage assay for BoNT/B using
both single and dual antibody assay procedures

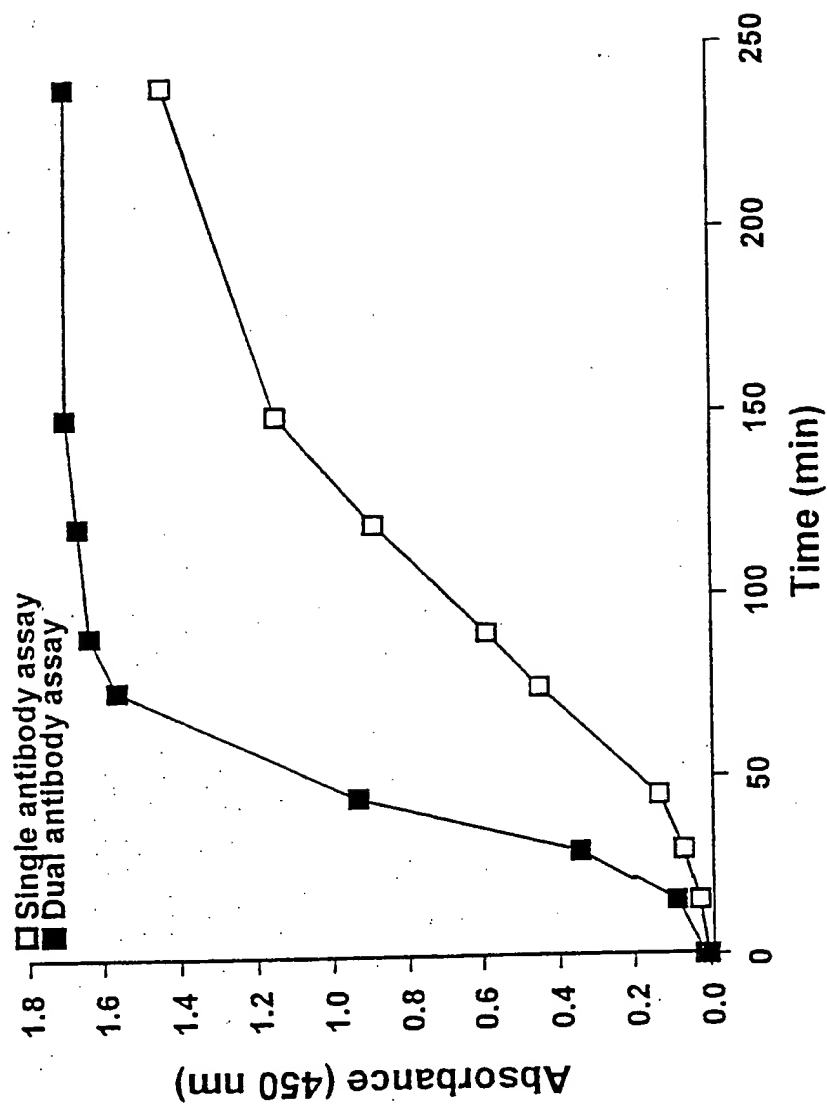


FIG. 4

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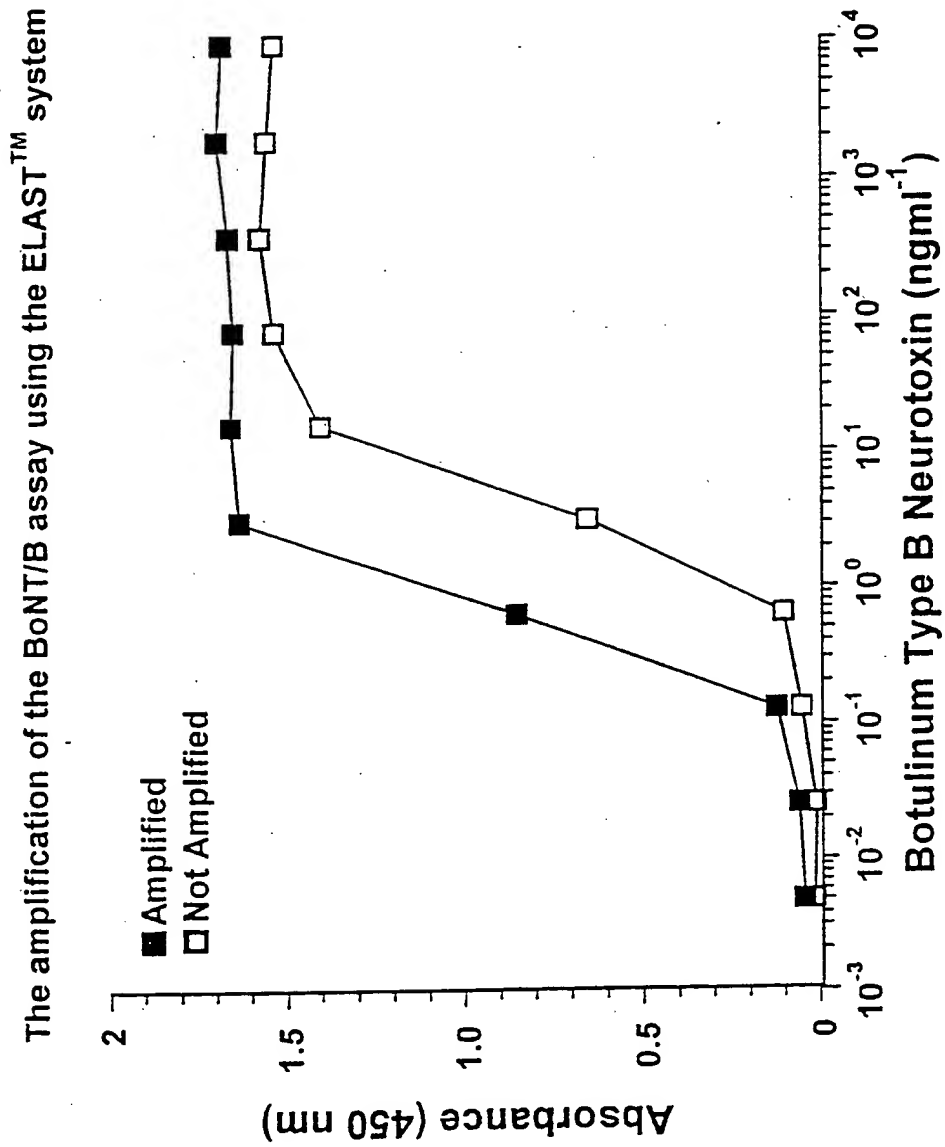


FIG. 5

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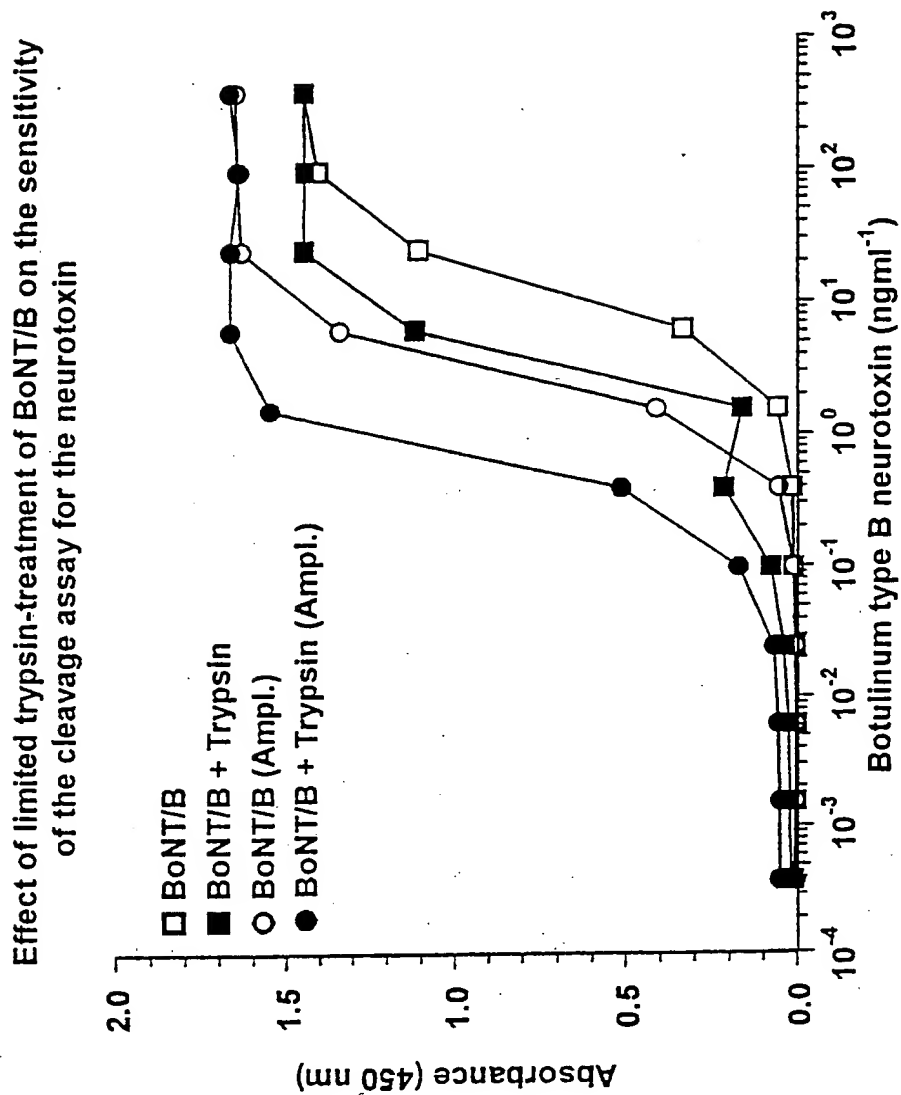


FIG. 6

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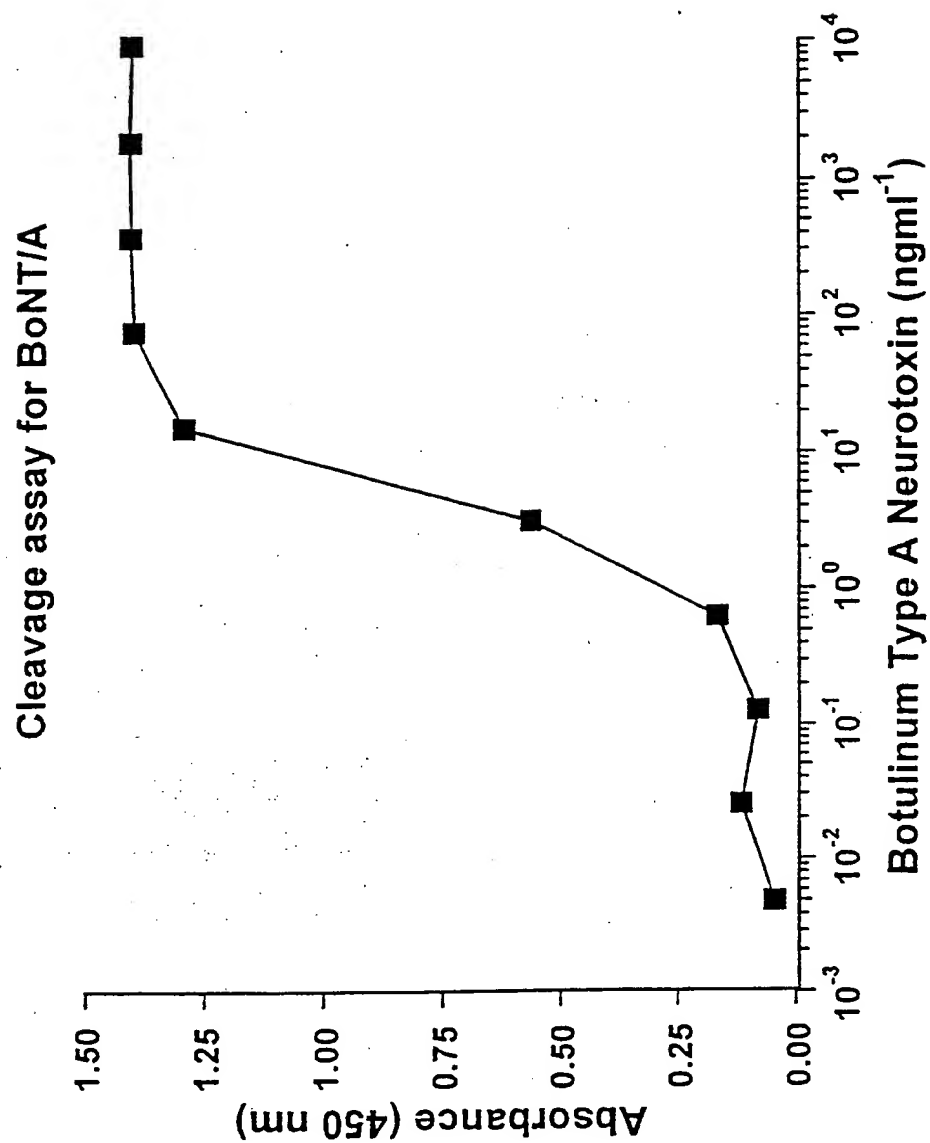


FIG. 7

INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 95/01279

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12Q1/37 C07K16/40 G01N33/68 G01N33/543 //G01N33/569

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 G01N C12Q C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US,A,5 171 662 (S. K. SHARMA) 15 December 1992 see abstract ---	1
A	EP,A,0 529 719 (MERCK & CO. INC.) 3 March 1993 see abstract ---	1
P,A	EUROPEAN JOURNAL OF BIOCHEMISTRY, vol. 225, no. 1, October 1994 pages 263-270, C .C SHONE ET AL. 'Peptide substrate specificity and properties of the zinc-endorpeptidase activity of botulinum type B neurotoxin.' --- -/--	

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents :

- *A* document defining the general state of the art which is not considered to be of particular relevance
- *E* earlier document but published on or after the international filing date
- *L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- *O* document referring to an oral disclosure, use, exhibition or other means
- *P* document published prior to the international filing date but later than the priority date claimed

- *T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- *X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- *Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- *Z* document member of the same patent family

Date of the actual completion of the international search

13 October 1995

Date of mailing of the international search report

24-10-95

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Authorized officer

Cartagena y Abella,P

INTERNATIONAL SEARCH REPORT

Int. Patent Application No

PCT/GB 95/01279

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,A	<p>BIOCHEMISTRY, vol. 33, 27 December 1994 pages 15365-15374, P. FORAN ET AL. 'Differences in the protease activities of Tetanus and Botulinum B toxins revealed by the cleavage of vesicle-associated membrane protein and various sized fragments.' ---</p>	
P,A	<p>NATURE, vol. 372, no. 1, 1 December 1994 pages 415-416, O. ROSSETTO ET AL. 'SNARE motif and neurotoxins.' -----</p>	

INTERNATIONAL SEARCH REPORT

Information on patent family members

Int. Patent Application No

PCT/GB 95/01279

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US-A-5171662	15-12-92	NONE	
EP-A-529719	03-03-93	US-A- 5274080	28-12-93
		US-A- 5229267	20-07-93
		US-A- 5276139	04-01-94
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